PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

(Chapter II of the Patent Cooperation Treaty)

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference HAEBE/P31526PC	**FOR FURTHER ACTION**	See Form PCT/IPEA/416
International application No. PCT/GB2004/004235	International filing date *(day/month/year)* 07.10.2004	Priority date *(day/month/year)* 07.10.2003
International Patent Classification (IPC) or national classification and IPC A61K47/48, A61P7/04		
Applicant UNIVERSITY OF LEICESTER et al.		

1. This report is the international preliminary examination report, established by this International Preliminary Examining Authority under Article 35 and transmitted to the applicant according to Article 36.
2. This REPORT consists of a total of 8 sheets, including this cover sheet.
3. This report is also accompanied by ANNEXES, comprising:
   a. ☒ *sent to the applicant and to the International Bureau)* a total of 24 sheets, as follows:
      ☒ sheets of the description, claims and/or drawings which have been amended and are the basis of this report and/or sheets containing rectifications authorized by this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions).
      ☐ sheets which supersede earlier sheets, but which this Authority considers contain an amendment that goes beyond the disclosure in the international application as filed, as indicated in item 4 of Box No. I and the Supplemental Box.
   b. ☐ *(sent to the International Bureau only)* a total of (indicate type and number of electronic carrier(s)) , containing a sequence listing and/or tables related thereto, in computer readable form only, as indicated in the Supplemental Box Relating to Sequence Listing (see Section 802 of the Administrative Instructions).

4. This report contains indications relating to the following items:
   - ☒ Box No. I Basis of the opinion
   - ☐ Box No. II Priority
   - ☒ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - ☐ Box No. IV Lack of unity of invention
   - ☒ Box No. V Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - ☒ Box No. VI Certain documents cited
   - ☐ Box No. VII Certain defects in the international application
   - ☒ Box No. VIII Certain observations on the international application

Date of submission of the demand	Date of completion of this report
21.04.2005	29.08.2005
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INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

International application No.
PCT/GB2004/004235

Box No. I Basis of the report

1. With regard to the **language**, this report is based on the international application in the language in which it was filed, unless otherwise indicated under this item.

   ☐ This report is based on translations from the original language into the following language , which is the language of a translation furnished for the purposes of:
   - ☐ international search (under Rules 12.3 and 23.1(b))
   - ☐ publication of the international application (under Rule 12.4)
   - ☐ international preliminary examination (under Rules 55.2 and/or 55.3)

2. With regard to the **elements*** of the international application, this report is based on *(replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report)*:

Description, Pages

1-7, 9-12, 17-22, 24-27, 29, 34-53	as originally filed
8, 13-16, 23, 27a, 28, 30-33	received on 10.01.2005 with letter of 06.01.2005

Sequence listings part of the description, Pages

1-7	received on 10.01.2005 with letter of 06.01.2005

Claims, Numbers

1-29	received on 12.07.2005 with letter of 11.07.2005

☒ a sequence listing and/or any related table(s) - see Supplemental Box Relating to Sequence Listing

3. ☐ The amendments have resulted in the cancellation of:
   - ☐ the description, pages
   - ☐ the claims, Nos.
   - ☐ the drawings, sheets/figs
   - ☐ the sequence listing *(specify)*:
   - ☐ any table(s) related to sequence listing *(specify)*:

4. ☐ This report has been established as if (some of) the amendments annexed to this report and listed below had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).
   - ☐ the description, pages
   - ☐ the claims, Nos.
   - ☐ the drawings, sheets/figs
   - ☐ the sequence listing *(specify)*:
   - ☐ any table(s) related to sequence listing *(specify)*:

* *If item 4 applies, some or all of these sheets may be marked "superseded."*

Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application,

☒ claims Nos. 19-29

because:

☒ the said international application, or the said claims Nos. 19-29 relate to the following subject matter which does not require an international preliminary examination (specify):

see separate sheet

☐ the description, claims or drawings *(indicate particular elements below)* or said claims Nos. are so unclear that no meaningful opinion could be formed *(specify)*:

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for the said claims Nos.

☐ the nucleotide and/or amino acid sequence listing does not comply with the standard provided for in Annex C of the Administrative Instructions in that:

the written form ☐ has not been furnished

☐ does not comply with the standard

the computer readable form ☐ has not been furnished

☐ does not comply with the standard

☐ the tables related to the nucleotide and/or amino acid sequence listing, if in computer readable form only, do not comply with the technical requirements provided for in Annex C-*bis* of the Administrative Instructions.

☐ See separate sheet for further details

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

International application No.
PCT/GB2004/004235

Box No. V Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-29
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-29
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	
	No:	Claims	see separate sheet

2. Citations and explanations (Rule 70.7):

see separate sheet

Box No. VI Certain documents cited

1. Certain published documents (Rule 70.10)

and / or

2. Non-written disclosures (Rule 70.9)

see separate sheet

Box No. VIII Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Supplemental Box relating to Sequence Listing

Continuation of Box I, item 2:

1. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application and necessary to the claimed invention, this report has been established on the basis of:

   a. type of material:

   ☒ a sequence listing

   ☐ table(s) related to the sequence listing

   b. format of material:

   ☒ in written format

   ☒ in computer readable form

   c. time of filing/furnishing:

   ☐ contained in the international application as filed

   ☐ filed together with the international application in computer readable form

   ☒ furnished subsequently to this Authority for the purposes of search and/or examination

   ☒ received by this Authority as an amendment on

2. ☒ In addition, in the case that more than one version or copy of a sequence listing and/or table(s) relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional observations, if necessary:

Concerning section III

Claims 19-29 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(I) PCT).

Concerning section V

1. The following documents are referred to; the numbering will be adhered to in the rest of the procedure:

   D1: WO 99/25383 A (QUADRANT HEALTHCARE LIMITED; CHURCH, NICOLA, JANE; HARRIS, ROY) 27 May 1999 (1999-05-27)
   D2: WO 98/17319 A (ANDARIS LIMITED) 30 April 1998 (1998-04-30)
   D3: WO 99/42146 A (QUADRANT HEALTHCARE LIMITED; HARRIS, ROY; MIDDLETON, SARAH, MARGARET) 26 August 1999 (1999-08-26)
   D4: LEVI M ET AL: "FIBRINOGEN-COATED ALBUMIN MICROCAPSULES REDUCE BLEEDING IN SEVERELYTHROMBOCYTOPENIC RABBITS" NATURE MEDICINE, NATURE PUBLISHING, CO, US, vol. 5, no. 1, January 1999 (1999-01), pages 107-111, XP002930943 ISSN: 1078-8956
   D5: DAVIES A R ET AL: "Interactions of platelets with SynthocytesTM, a novel platelet substitute" PLATELETS (ABINGDON), vol. 13, no. 4, June 2002 (2002-06), pages 197-205, XP009043550 ISSN: 0953-7104
   D6: EP-A-0 618 225 (MERCK PATENT GMBH) 5 October 1994 (1994-10-05)

   Unless indicated otherwise reference is made to the relevant passages emphasized in the search report.

2. The document D1 discloses albumin microparticles linked to fibrinogen via a spacer which can be a peptide which allows for the fibrinogen to bind to GPIIb/GPIIIa, i.e. activated platelets (p. 2, lines 22-25). The microparticles are used in the treatment of

thrombocytopenia. The peptide has no affinity for fibrinogen and is covalently linked to fibrinogen, therefore D1 does not anticipate the subject-matter of the claims.

The document D2 discloses albumin microparticles linked to fibrinogen via a tetra-alanine spacer, wherein the spacer allows for the fibrinogen to bind to GPIIb/GPIIIa, i.e. activated platelets, for the treatment of thrombocytopenia, wound, bleeding. However, the spacer has no affinity for fibrinogen and is covalently linked to fibrinogen, therefore D2 does not anticipate the subject-matter of the claims.

The documents D3 to D5 disclose albumin microparticles covalently (D3) or non-covalently (D4, D5) coated with fibrinogen, for the treatment of thrombocytopenia. The fibrinogen is not linked to the microparticles via a peptide having affinity for fibrinogen, thereby not anticipating the subject-matter of the claims.

Hence, the present application meets the criteria of Article 33(1) PCT, because the subject-matter of the claims appears to be new in the sense of Article 33(2) PCT.

2. The closest prior art is the documents D2 from the present inventors which discloses albumin microparticles covalently linked with fibrinogen via a tetra-alanine spacer. The application differs from D2 in the choice of the spacer.
The problem of the application is to provide alternative albumin-fibrinogen microparticles for treating thrombocytopenia, the solution being the use of a spacer derived either from GPIIb/GPIIIa (claims 3, 4) or from fibrin (claims 5, 6).

The experiments of the application compare 3 type of microparticles:
- product 1: albumin-fibrinogen linked via a fibrin derived peptide (invention).
- product 2: albumin-fibrin derived peptide without fibrinogen.
- reference product: fibrinogen covalently linked to albumin microparticles.

The product of the application shows that the reference product is aggregated less effectively by thrombin, indicating a less favourable configuration of the fibrinogen due to the covalent link (Table 4). The product of the application further proves to aggregate specifically the activated platelets whereas the reference product also aggregates inactive platelets (Table 6). This surprising technical effects could not be

foreseen by the skilled person, hence the subject-matter of the claims appears to make an inventive contribution to the art.

3. For the assessment of the present claims 19-29 on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

Concerning section VI

The document WO 2004/045542 A cited as P-document in the search report might be relevant for the question of novelty upon entry in the regional phases.

Concerning section VIII

Claims 1, 2, 7-29 do not meet the requirements of Article 6 PCT in that the matter for which protection is sought is not clearly defined. The claims attempt to define the subject-matter in terms of the result to be achieved ("a peptide having affinity for fibrinogen, the peptide being capable of non-covalently binding fibrinogen"), which merely amounts to a statement of the underlying problem, without providing the technical features necessary for achieving this result. In the present case, it appears that the only peptide capable of fulfilling this requirement is the one defined by claims 3 to 6.

Description of the Invention

The present invention provides an injectable pharmaceutical product comprising an agent, the agent comprising an insoluble carrier to which is bound a peptide, the peptide being capable of binding fibrinogen such that the agent binds via the bound fibrinogen to activated platelets in preference to inactive platelets, and wherein the peptide is not fibrinogen.

In one embodiment, the peptide binds to the region of fibrinogen that is naturally bound either by the platelet membrane glycoproteins GPIIb-IIIa or by fibrin.

In a preferred embodiment, the peptide binds to the region of fibrinogen that is naturally bound by GPIIb-IIIa. The binding of GPIIb-IIIa to fibrinogen is discussed in Bennett, 2001, *Annals of NY Acad. Sci.*, **936**, 340-354.

The peptide may bind to one or both of the carboxy- or amino-terminal domains of the α-chain of fibrinogen. More particularly, the peptide may bind to an RGD-containing motif in one or both of said domains. The RGD-containing motif may have the sequence RGDX (SEQ. ID NO: 1), where X is any amino acid, such as serine, valine, phenylalanine or alanine, and thus may be RGDF (SEQ. ID NO: 2) at amino acids 95-98, or RGDS (SEQ. ID NO: 3) at amino acids 572-575.

The peptide may bind to the C-terminal domain of the γ-chain of fibrinogen. More particularly the peptide may bind to a sequence within the final 15, 12, 10 or 4 amino acids of the C-terminal domain of the fibrinogen γ-chain. The final 12 amino acids are usually HHLGGAKQAGDV (SEQ. ID NO: 4)

In another preferred embodiment, the peptide binds to the region of fibrinogen that is naturally bound by fibrin. Fibrin binding to fibrinogen is

Thus the fibrinogen-binding peptide may comprise the sequence of AVTDVNGDRHDLLVGAPLYM (SEQ. ID NO: 5), which represents the sequence of amino acids 294-314 of GPIIb, or a fibrinogen-binding fragment thereof. Such fragments include the sequence TDVNGDGRHDL (SEQ. ID NO: 6) (296-306), the sequence GDGRHDLLVGAPL (SEQ. ID NO: 7) (300-312) and the terminal tetrapeptide ~~GALP~~ GAPL (SEQ. ID NO: 8). These sequences are thought to be involved in the binding of fibrinogen and, in particular, the γ-chain of fibrinogen (Bennett, 2001, *op. cit.*; D'Souza *et al*, 1991, *Nature*, **350**, 66-68; Taylor & Gartner, 1992, *J. Biol. Chem.*, **267**, 11729-33). The similar effects of fragments 296-306 and 300-312 suggest that fragment 300-306 may also provide fibrinogen-binding activity.

Grunkemeier *et al* (1996, *J. Molecular Recognition*, **9**, 247-257) reported that purified TDVNGDGRHDL (SEQ. ID NO: 6) (designated "B12") peptide caused inhibition of platelet aggregation. Grunkemeier *et al* used this information to propose non-platelet-adhesive materials coated in B12 peptide, and hypothesised that B12 would bind fibrinogen specifically in the region that binds to the GPIIb/IIIa platelet receptor, thus blocking platelet aggregation. Therefore, the understanding in Grunkemeier *et al* is that, when immobilised, the B12 peptide can be used to block fibrinogen binding to platelets, and thus inhibit platelet aggregation. In light of this teaching, it was not apparent that the B12 peptide would be suitable for use in a platelet substitute for aiding platelet aggregation and blood clot formation.

The fibrinogen-binding peptide may comprise one or more of the peptides APLHK (SEQ. ID NO: 9), EHIPA (SEQ. ID NO: 10) and GAPL (SEQ. ID NO: 8) which were shown in Gartner, 1991, *Biochem. Biophys. Res. Commun.*, **180**(3), 1446-52 to be hydropathically equivalent peptide mimics of the fibrinogen binding domain of GPIIb-IIIa.

The fibrinogen-binding peptide may comprise the sequence of residues 95-223 of GPIIIa or a fibrinogen-binding fragment thereof. For example, residues 211-222, comprising the sequence SVSRNRDAPEGG (SEQ. ID NO: 11) is thought to be an important fibrinogen-binding domain in GPIIIa (Charo *et al*, 1991, *J. Biol. Chem.*, 266, 1415-1421).

Other suitable regions of GPIIIa include residues 109-171 and 164-202.

The skilled person will appreciate that fragments or variants of any of these sequences may also be used, so long as they provide fibrinogen-binding activity according to the present invention.

A particularly preferred fibrinogen-binding peptide comprises a sequence obtained from the platelet membrane glycoprotein GPIIb, namely TDVNGDGRHDL (SEQ. ID NO: 6), or a variant of such a sequence.

Variants of TDVNGDGRHDL (SEQ. ID NO: 6) include –

T(D,E)VNG(D,E)GRH(D,E)L (SEQ. ID NO: 12)

TD(V, L)NGDGRHDL (SEQ. ID NO: 13)

TDV(N,Q)GDGRHDL (SEQ. ID NO: 14)

TDVNGDG(R,K)HDL (SEQ. ID NO: 15)

Such variants will have substantially the same fibrinogen binding activity as TDVNGDGRHDL (SEQ. ID NO: 6), in that they will have substantially the same affinity for fibrinogen and, when bound, fibrinogen will have substantially the same conformation and activity as when bound to TDVNGDGRHDL (SEQ. ID NO: 6). By "substantially the same fibrinogen-binding activity" we include variants that bind fibrinogen with an affinity up to 1, 2, 3, 4, 5, 10, 50, 100 or more orders of magnitude different (either higher or lower) to TDVNGDGRHDL (SEQ. ID NO: 6). Lower numbers are preferred.

Kuyas *et al*, 1990, *Thrombosis and Haemostasis*, 63(3), 439, describes the use of the synthetic peptide GPRPK (SEQ. ID NO: 16), immobilised via the C-terminal lysine to fractogel, to isolate fibrinogen from human plasma. Kuyas *et al* explains that human fibrinogen has a strong affinity for fibrin, and reports that the authors utilised a peptide comprising the N-terminal sequence of the α-chain of fibrin exposed by the action of thrombin, GPRP (SEQ. ID NO: 17), which had been shown to bind fibrinogen (Laudano & Doolittle, 1980, *Biochemistry*, **19**, 1013; Laudano *et al*, 1983, *Ann. N.Y. Acad. Sci.*, **408**, 315). Kuyas *et al* concludes that the 'core' sequence GPR is required for fibrinogen binding.

Thus, the fibrinogen-binding peptide as used in the product may comprise the sequence of a fibrinogen-binding region of fibrin such as the N-terminal region of the α-chain or the C-terminal region of the β-chain. Accordingly the peptide may have the sequence Gly-(Pro/His/Val)-Arg-Xaa (SEQ. ID NO: 18) at the amino terminus, wherein Xaa is any amino acid. In this context, by "at the amino terminus" we mean that the Gly residue in the above tetrapeptide sequence should represent the first amino acid of the peptide when read from the N-terminus to the C-terminus. By "Pro/His/Val" we mean that either proline, histidine or valine is included at that position. In one embodiment, proline and histidine are preferred, and proline is most preferred.

Kuyas *et al* fails to disclose an injectable pharmaceutical product according to the present invention because the peptide is bound to Fractogel. Fractogel is composed of polymethacrylate and has a minimum particle size of 20mm and would therefore not be pharmaceutically acceptable.

The peptide may comprise the sequence of Gly-Pro-Arg-Pro (SEQ. ID NO: 17) at the amino terminus.

Alternatively, the peptide may comprise the sequence of Gly-Pro-Arg-Sar (SEQ. ID NO: 19 (Sar is short for sarcosine, which is methyl glycine), Gly-Pro-Arg-Gly (SEQ. ID NO: 19) or Gly-Pro-Arg-Val at the amino terminus.
(SEQ. ID NO: 19)

The peptide may comprise, in addition to a fibrinogen-binding sequence, an amino acid or sequence designed to aid attachment of the peptide to the carrier. For example, the peptide may include a terminal cysteine for linking to a thiol reactive group on the carrier (see below).

Typically the peptide has from 4 to 200 amino acids. Preferably, the peptide is no more than 150, 100, 90, 80, 70, 60, 50, 40, 30 or 20 amino acids in length. Preferably, the peptide is at least 4, 5, 6, 7, 8, 9, 10, 11 or more amino acids in length, although the minimum length should be at least long enough to include the fibrinogen-binding sequence in full.

The peptide may also comprise a spacer sequence. This can provide for spatial distances between the fibrinogen-binding sequence and the linkage to the carrier. This may aid in preserving the fibrinogen-binding activity of the peptide. For example, a spacer sequence of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more amino acids may be suitable. The sequence of the spacer may comprise a mix of amino acids or be a repeat of a single amino acid. For example, a poly(glycine) sequence may be suitable for use as a spacer.

The carrier should be insoluble, inert and biocompatible. The carrier should exhibit an insignificant effect on blood coagulation tested by adding the carrier to plasma and demonstrating no effect on the activated partial thromboplastin clotting time (APPT) using for example micronized kaolin (supplied by Helena Laboratories Ltd.) to activate recalcified plasma or the prothrombin clotting time (PT) using for example Manchester

Accordingly, the product may additionally comprise fibrinogen, or a variant or fragment thereof, having an inducible platelet-aggregating activity, bound to the said peptide.

By "inducible platelet-aggregating activity", we mean that the fibrinogen binds to activated platelets in preference to inactive platelets. Preferably, if administered to a patient intravenously, the fibrinogen portion of the product will preferentially become involved in formation of a blood clot at the site of a wound where platelets are already activated. Methods for determining whether the platelet-aggregating activity of fibrinogen is "inducible" are discussed above.

The source of the fibrinogen can be, for example, a purified protein derived from plasma or blood or from a recombinant source. The fibrinogen may be human or non-human in sequence.

Any variant or fragment of fibrinogen may be used, provided that it has a useful level of inducible platelet-aggregating activity. In this context, a useful level of inducible platelet-aggregating activity means that the variant or fragment can be used with the product of the invention to cause aggregation of activated platelets in preference to inactive platelets, as described above. Preferably, any such variant or fragment includes residues 398-411 of the gamma chain of fibrinogen. In a preferred embodiment, the variant or fragment may include, or even consist of, HHLGGAKQADV (SEQ. ID NO: 20)

Accordingly, the present invention also provides an injectable pharmaceutical product having an inducible platelet-aggregating activity comprising an insoluble carrier to which fibrinogen, or a variant or fragment thereof, is bound in a configuration such that the fibrinogen (or variant or fragment) binds to activated platelets in preference to inactive platelets.

Schedule of SEQ ID Nos.

1. RGDX where X is any amino acid
2. RGDF
3. RGDS
4. HHLGGAKQAGDV
5. AVTDVNGDRHDLLVGAPLYM
6. TDVNGDGRHDL
7. GDGRHDLLVGAPL
8. GAPL
9. APLHK
10. EHIPA
11. SVSRNRDAPEGG
12. T(D,E)VNG(D,E)GRH(D,E)L
13. TD(V,L)NGDGRHDL
14. TDV(N,Q)GDGRHDL
15. TDVNGDG(R,K)HDL
16. GPRPK
17. GPRP
18. G(P,H,V)RX where X is any amino acid
19. GPR(X,G,V) where X is methylglycine (sarcosine)
20. HHLGGAKQADV
21. GPRPC
22. GPRPGGGC
23. GPRPGGGGGGC
24. G(P,H)RX where X is any amino acid
25. G(P,H)R(P,X,G,V) where X is methylglycine (sarcosine)

Example 1: Production of fibrinogen-linked particles (FLPs) and measurement of bound fibrinogen

1. General method for producing Fibrinogen linked microspheres, using the peptide GPRP with a spacer consisting of 0, 3 or 6 glycine residues.

(i) A 10mg albumin/ml suspension of washed human albumin microspheres was prepared in an isotonic buffer at pH 7.4 (e.g. phosphate buffered saline). The albumin microspheres used were of a diameter suitable for intravenous use and therefore less than 6 μm. Suitable albumin microspheres are known in the art. For example, WO 03/015756 discloses albumin microparticles that have a diameter of 2-3 μm, pH 7.4.

(ii) 30μl of 10mM 5,5'-Dithio-bis(2-Nitrobenzoic acid) (DTNB) was added to 10mg of microspheres and mixed at room temperature for 2 hours. The microspheres were then separated from the supernatant and washed at least twice in buffer (we used phosphate buffered saline), using centrifugation at 3000 × *g* for at least 5 minutes.

(iii) The pellet was resuspended in 1 ml of a suitable buffer such as phosphate buffered saline. The peptide (either GPRPC (SEQ. ID NO: 21) GPRPGGGC (SEQ. ID NO: 22) or GPRPGGGGGGC (SEQ. ID NO: 23)), supplied by Merck Biosciences, was dissolved in phosphate buffered saline and added to the microspheres at a final concentration of 0.23mM. This was mixed at room temperature for 24 hours. An appropriate negative control was provided by treating the preparation with 0.23mM L-cysteine (cysteine control). A wash was performed by centrifuging at 3000 × *g* for 5 minutes and resuspending in phosphate buffered saline. The

(iii) Data were recorded as the percentage of microspheres positive for fibrinogen and the median fluorescence intensity of the particles. The latter is a measure of the amount of fibrinogen bound to the microspheres and is in arbitrary units (log scale).

The products made by method 1 above were tested using method 2. Specifically the products tested had, as the bound peptide, either "peptide Gly^n=0" (i.e. GPRPC (SEQ. ID NO: 21)), "peptide Gly^n=3" (i.e. GPRPGGGC (SEQ. ID NO: 22)) or "peptide Gly^n=6" (i.e. GPRPGGGGGGC (SEQ. ID NO: 23)). A cysteine control sample was also included. The results are reported in Table 1 below.

Table 1: Fibrinogen binding to peptide-linked microspheres

Flow cytometric measure	**Peptide $Gly^n = 0$**	**Peptide $Gly^n = 3$**	**Peptide $Gly^n = 6$**	**Cysteine control**
Percent positive	6	94	95	32
Median fluorescence intensity	2.4	28	26	0.9

The results show that the ability of a peptide comprising a GPRP N-terminal sequence to bind fibrinogen can be modified by inclusion of a spacer between the peptide and the microsphere. Without the spacer only 6% of the microspheres carry fibrinogen but with a spacer consisting of 3 or 6 glycine residues, greater than 90% of the microspheres bind fibrinogen.

Example 2: Further analysis of fibrinogen-linked particles

The following products were produced –

Product 1: Artificial Platelets were produced, using the peptide GPRPGGGGGGC (SEQ. ID NO: 23) (i.e. $Gly^n = 6$) with bound fibrinogen, using steps (i) to (iv) of method 1 of Example 1, as discussed above, except that step (iv) used 0.1 mg/ml of fibrinogen, rather than 3 mg/ml.

Product 2: Artificial Platelets were produced, using the peptide GPRPGGGGGGC (SEQ. ID NO: 23) (i.e. $Gly^n = 6$) *without fibrinogen*, using steps (i) to (iii) of method 1 as discussed above.

A reference batch: A reference batch of microspheres was prepared using the method described in WO 98/17319. Specifically:

(i) 1gm of microspheres were washed and suspended in 50ml 0.01M sodium phosphate buffer, pH 6.0.

(ii) A vial of freeze-dried fibrinogen (for example the material supplied by the Scottish National Blood Transfusion Service) was reconstituted.

(iii) Fibrinogen was diluted to 10mg/ml using 0.01M sodium phosphate buffer at pH 6.0.

(iv) 15ml of dilute fibrinogen solution was added per gram of microspheres, and mixed for 4 hours at room temperature (e.g. 20°C).

(v) The mixture was then centrifuged 2000 × *g* for 15 minutes at 20°C to pellet the microspheres. The supernatant was removed and the pellet washed in 0.01M sodium phosphate pH 6.0.

(vi) The mixture was then centrifuged and resuspended in 0.01M sodium phosphate buffer, at least twice until the supernatant was low in protein as determined by absorbance at E280.

(vii) The microspheres were then finally washed and resuspended at 10mg/ml in phosphate buffered saline pH 7.4.

(viii) Fibrinogen coated microspheres were then filled into 500ul aliquots and stored at minus 70°C.

In summary, product 1 is a FLP (fibrinogen-linked particle) of the invention having fibrinogen bound via the peptide GPRPGGGGGGC (SEQ. ID NO. 23), the peptide being bound to the microsphere. Product 2 is identical to product 1 except that it has no fibrinogen bound to the peptide GPRPGGGGGGC (SEQ. ID NO. 23). The reference product has fibrinogen bound directly to the surface of the microsphere, in the manner known from the prior art, such as in WO 98/17319 and Davies *et al*, *supra*.

These products were tested for fibrinogen binding using method 2 of Example 1, above. The results are reported in table 2 below.

Table 2: Fibrinogen binding to Product 1. Product 2 and Reference Batch of microspheres

Flow cytometric measure	Product 1	Product 2	Reference batch
Percent positive	75	13.1	94
Median fluorescence intensity	4.6	1.6	22

These results demonstrate that the relative fluorescence of the fibrinogen-coated microspheres was greater than that of microspheres that had not been incubated with fibrinogen. Product 1 shows lower percentage positive and MFI values than for the Gly^n=6 product described in Table 1. Without being bound by theory, we believe that the difference is as a result of the lower fibrinogen concentration used in the production of Product 1.

To determine the effect of fibrinogen concentration on activity of products of the invention, microspheres were prepared using DNTB to link GPRPGGGGGGC (SEQ. ID NO: 23) according to the method described above.

To 1 ml of microspheres obtained from step (iii) of method 1 of Example 1, 1 ml of fibrinogen at 1 mg/ml, 0.1 mg/ml or 0.01 mg/ml was added and mixed for 1 hour at room temperature. A zero concentration control was included.

A reference batch control, having fibrinogen covalently linked to the microsphere surface (prepared in the manner discussed above) was also included in the analysis.

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CLAIMS

1. An injectable pharmaceutical product comprising an agent, the agent comprising an insoluble carrier to which is bound a peptide, the peptide being capable of binding fibrinogen such that the agent binds via the bound fibrinogen to activated platelets in preference to inactive platelets, and wherein the peptide is not fibrinogen.

2. A product according to Claim 1 in which, if the product is introduced intravenously, the peptide binds fibrinogen such that the bound fibrinogen will preferentially become involved in formation of a blood clot at the site of a wound where platelets are already activated.

3. A product according to Claim 1 or 2 wherein the peptide comprises a fibrinogen-binding sequence obtained from the platelet membrane glycoprotein GPIIb or GPIIIa, such as the sequence TDVNGDGRHDL or a variant of such a sequence.

4. A product according to any one of the preceding claims wherein the peptide comprises TDVNGDGRHDL

5. A product according to any one of the preceding claims wherein the peptide comprises the sequence of Gly-(Pro/His)-Arg-Xaa at the amino terminus, wherein Xaa is any amino acid.

6. A product according to Claim 5 wherein Xaa is Pro, Sar, Gly or Val.

7. A product according to any one of the preceding claims wherein the peptide has from 4 to 200 amino acids.

8. A product according to any one of the preceding claims wherein the carrier has a size suitable to ensure transmission of the agent through the lung capillary bed.

9. A product according to any one of the preceding claims wherein the carrier is a microparticle.

10. A product according to Claim 11 wherein the microparticle is a protein microparticle, such as an albumin microparticle.

11. A product according to any one of Claims 8 to 10 wherein the wherein the product comprises a population of carriers of which less than 2% are in excess of 6 μm as a maximum dimension.

12. A product according to any one of Claims 8 to 11 wherein the majority of carriers are from 2 to 4 μm as a maximum dimension.

13. A product according to any one of the preceding claims wherein the peptide is bound to the carrier by a covalent bond.

14. A product according to Claim 13 wherein the peptide comprises a cysteine and is bound to the carrier by linking the -SH group of the cysteine to a thiol reactive group on the carrier.

15. A product according to any one of the preceding claims wherein the product additionally comprises fibrinogen, or a variant or fragment thereof, having an inducible platelet-aggregating activity, non-covalently bound to the said peptide.

~~16. A product according to Claim 15 wherein the fibrinogen (or variant or fragment) is bound to the peptide by non-covalent bonds.~~

~~17. A product according to Claim 15 or 16 wherein the fibrinogen (or variant or fragment) is bound to the peptide by covalent bonds.~~

(X) , via a peptide having an affinity for fibrinogen,

16
~~18.~~ An injectable pharmaceutical product having an inducible platelet-aggregating activity comprising an insoluble carrier to which fibrinogen, or a variant or fragment thereof, is bound (non-covalently (X)) in a configuration such that the fibrinogen binds to activated platelets in preference to inactive platelets.

17 16
~~19.~~ A product according to Claim ~~18~~ which, when introduced intravenously, will only become involved significantly in formation of a blood clot at the site of a wound where platelets are already activated.

18
~~20.~~ A method for preparing a product as defined in any one of Claims 15 to 17
~~19~~, comprising providing a product according to any one of Claims 1 to 14 and mixing with fibrinogen, or a variant of fragment thereof and optionally further comprising one or more of the following steps –

(a) removing unbound fibrinogen;

(b) formulating the product with a pharmaceutically acceptable carrier or diluent;

(c) diluting the product to provide a pharmaceutically acceptable unit dose; and

(d) sterilising the product, or ensuring product sterility throughout steps (a) to (c).

19 21. A method of promoting haemostasis in an individual comprising administering to the individual a pharmaceutically effective dosage of a product as defined in any one of Claims 1 to 17 ~~19~~.

20 22. A method of treating an individual with thrombocytopenia comprising administering a pharmaceutically effective dosage of a product as defined in any one of Claims 1 to 17 ~~19~~.

21 23. A product as defined in any one of Claims 1 to 17 ~~19~~ for use in medicine.

22 24. Use of a product as defined in any one of Claims 1 to 17 ~~19~~ in the manufacture of a medicament for promoting haemostasis.

23 25. Use of a product as defined in any one of Claims 1 to 17 ~~19~~ in the manufacture of a medicament for the treatment of a patient with thrombocytopenia.

24 26. A method or use according to any one of Claims 19 ~~21~~ to 23 ~~25~~ wherein the patient has a platelet count below $400x10^9/l$, preferably below $150x10^9/l$.

25 27. A method or use according to Claim 24 ~~26~~ wherein the platelet count is below $10x\ 10^9/l$.

26 28. A method or use according to any one of Claims 19 ~~21~~ to 25 ~~27~~ wherein the patient has a failure in platelet production from the bone marrow.

27 26
29. A method or use according to Claim 28 wherein the failure in platelet production from the bone marrow is caused by a blood cancer, or cytotoxic chemotherapy or radiotherapy.

28 19 21 22
30. A method or use according to any one of Claims 21, 23 or 24 wherein the patient has an inherited or drug-induced disorders in platelet number or function.

29 19 21 22
31. A method or use according to any one of Claims 21, 23 or 24 wherein the patient's platelets have been mechanically damaged.